·Original Article·

$A\beta_{31-35}$-induced neuronal apoptosis is mediated by JNK-dependent extrinsic apoptosis pathway

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Abstract: Objective To investigate whether JNK-caspase-dependent apoptotic pathway is involved in $A\beta_{31-35}$-induced apoptosis of cultured cortical neurons. **Methods** Cultured cortical neurons were treated with $A\beta_{31-35}$ (25 μmol/L) for 4 h, 8 h, 16 h and 24 h, respectively. Caspase activities were measured using a spectrophotometer. Levels of c-Jun phosphorylation (p-c-Jun) and Fas ligand (FasL) expression were assessed by immunocytochemistry method and quantified using Image-pro plus11.0 image processing and analysis software. **Results** Treatment with $A\beta_{31-35}$ (25 μmol/L) for 24 h induced significant increases in the activities of caspase-3 and caspase-8 in the cortical neurons. Besides, $A\beta_{31-35}$ could time-dependently enhance the expression of p-c-Jun protein. Moreover, SP600125 application (100 nmol/L) could completely abolish $A\beta_{31-35}$ neurotoxicity. The increase in FasL expression was detected at 8 h, 16 h and 24 h after $A\beta_{31-35}$ treatment, and SP600125 (100 nmol/L) significantly inhibited FasL expression. **Conclusion** JNK-c-Jun-FasL-caspase-dependent extrinsic apoptotic pathway plays a critical role in mediating $A\beta_{31-35}$-induced apoptosis of cultured neurons.

Keywords: $A\beta_{31-35}$; neurotoxicity; caspase; JNK pathway

1 Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder that is characterized by senile plaque, neurofibrillary tangle, and neuronal loss[1,2]. Massive and progressive neuronal death is a key pathological feature of AD. The importance of Aβ in the pathogenesis of AD has been suggested by several findings. Thus, it is important to determine the mechanism by which Aβ induces neuronal cell death.

It has long been recognized that $A\beta_{1-42}$ induces apoptotic cell death in cortical neurons in AD brains and cultured neurons[3,4]. Our previous studies and other reports have shown that even $A\beta_{31-35}$, a smaller β-amyloid peptide fragment, can also induce signs of apoptosis in cultured neurons[5,6]. Some events have been implicated in $A\beta_{31-35}$-induced apoptosis, including the perturbation of intracellular calcium homeostasis[7] and the up-regulation of the cAMP-signaling pathway[8]. However, the specific molecular mechanisms have not been well defined.

Currently, 2 apoptotic pathways are relatively well understood at the molecular level: the extrinsic (the death receptor pathway) and the intrinsic (mitochondrial pathway). The extrinsic and intrinsic pathways were initially considered to be independent. However, it is now clear that a cross-talk exists between the 2 pathways, which is mediated by caspase, ultimately leading to cell apoptosis[9,10].

JNK is an important intermediary in signaling pathways that transduce extracellular stimulation into intracellular responses. JNK has been implicated in a number of physiological processes, including cell growth, differentiation and apoptosis[11-14]. These facts suggest a role of the JNK pathway in $A\beta_{31-35}$-induced apoptosis. However, the relationship

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between JNK and the cytotoxic signal of Aβ_{31-35} is not clear. The purpose of this study was to investigate this relationship.

2 Materials and methods

2.1 Materials Aβ_{31-35} was obtained from Sigma. Caspases (caspase-3 and caspase-8), Apoptosis Detection, Colorimetric BioGene kit was purchased from USBiological (Swampscott, MA, USA). Anti-Fas ligand (FasL) and anti-p-c-Jun antibodies were both from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.2 Cell culture conditions Cortical neurons in primary culture were obtained from newborn Sprague-Dawley rats (< 3 d) as described previously. Dissociated cells were plated on coverslips or in plastic dishes precoated with poly-*L*-lysine. Neurons were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% heat-inactived fetal bovine serum, 4 mmol/L glutamine, and 4.5 g/L *D*-glucose in a humidified CO_2 incubator at 37 ºC. Non-neuronal cell division was inhibited by Cytarabine treatment for 24 h. The experiments were performed at day 7-8 in culture. By this method, the purity of neurons was >95% as detected by immunostaining with MAP2.

2.3 Treatment of neurons Stock solution of Aβ_{31-35} (2.5 mmol/L) was prepared in DMEM and stored at -20 ºC. To prepare the aggregated Aβ_{31-35} peptides, the same volume of PBS was added to the stock solution and incubated at 37 ºC for 3-5 d. The final concentration of 25 μmol/L was used for Aβ_{31-35} application, since it could induce neurotoxicity during cell culture, as previously determined by our lab group[8].

2.4 Measurement of caspase activity The activities of caspase-3 and caspase-8 were measured using Caspases (caspase-3, 8), Apoptosis Detection, Colorimetric BioGene kit according to the manufacturer's protocol. The assay is based on the spectrophotometric detection of the chromophore pNA after cleavage from the labeled substrate DEVD-pNA and IETD-pNA, respectively. The pNA light emission was quantified at 405 nm using a spectrophotometer.

2.5 Immunocytochemistry technique For immunocytochemistry detection, cells were fixed in 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, and blocked with 3% BSA. Cells were incubated with primary antibodies (anti-FasL, 1:80; anti-p-c-Jun, 1:100), and then incubated with HRP-conjugated secondary antibody (1:1 000; Sigma). Finally, the color was developed with diaminobenzidine (DAB). The densities were quantitated by measuring the intensity and the area of positive region using Image-pro plus11.0 image processing and analysis software (Olympus, Japan). The results were described by the mean densities.

2.6 Statistical analysis All the experiments in the present study were repeated at least 3 times in independent treatment. Data were presented as mean±SD. Statistical analysis was performed using one-way ANOVA. $P < 0.05$ was considered as significantly different.

3 Result

3.1 Aβ_{31-35} induced significant increases in the activities of caspase-3 and caspase-8 in cortical neurons Our previous studies and other reports have demonstrated that treatment with Aβ_{31-35} (25 μmol/L) for 24 h could induce apoptosis of cultured neurons. To investigate the involvement of caspase-dependent and caspase-independent pathways in Aβ_{31-35}-induced apoptosis, the primary cultures of rat cortical neurons were treated with Aβ_{31-35} (25 μmol/L) for 24 h, and activities of caspase-3 and caspase-8 were assessed at 405 nm by using a spectrophotometer. As shown in Fig. 1, Aβ_{31-35} induced significant increases in the activities of caspase-3 (0.112±0.009 with Aβ_{31-35} treatment *vs* 0.024±0.003 in control, $P < 0.05$) and caspase-8 (0.02±0.001 with Aβ_{31-35} treatment *vs* 0.005±0.0008 in control, $P < 0.05$) in the cortical neurons. The results imply that Aβ_{31-35}-induced apoptosis is mediated by caspase-dependent extrinsic apoptotic pathways.

3.2 Aβ_{31-35} activated the JNK pathway in cortical neurons To test the hypothesis that the JNK signaling pathway may be involved in Aβ_{31-35}-induced apoptosis of cortical neurons, the activation of the component of the JNK pathway during Aβ_{31-35} treatment was investigated. The cortical neurons were treated with Aβ_{31-35} (25 μmol/L) for 1 h, 4 h, 8 h, 16 h and 24 h, respectively, and the total amount of p-c-Jun protein was assessed using antibody against p-c-Jun (at the site of Ser73). As shown in Fig. 2, expression of p-c-Jun in treated cortical neurons was enhanced in a time-dependent way. The

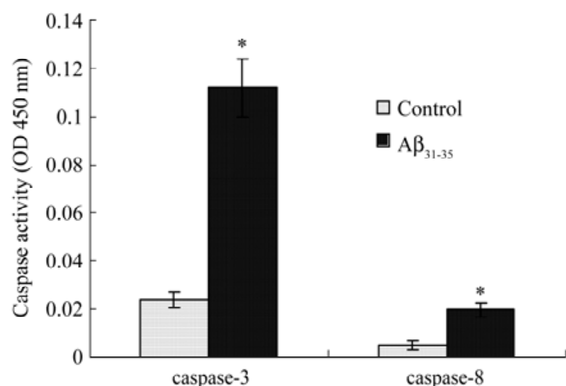
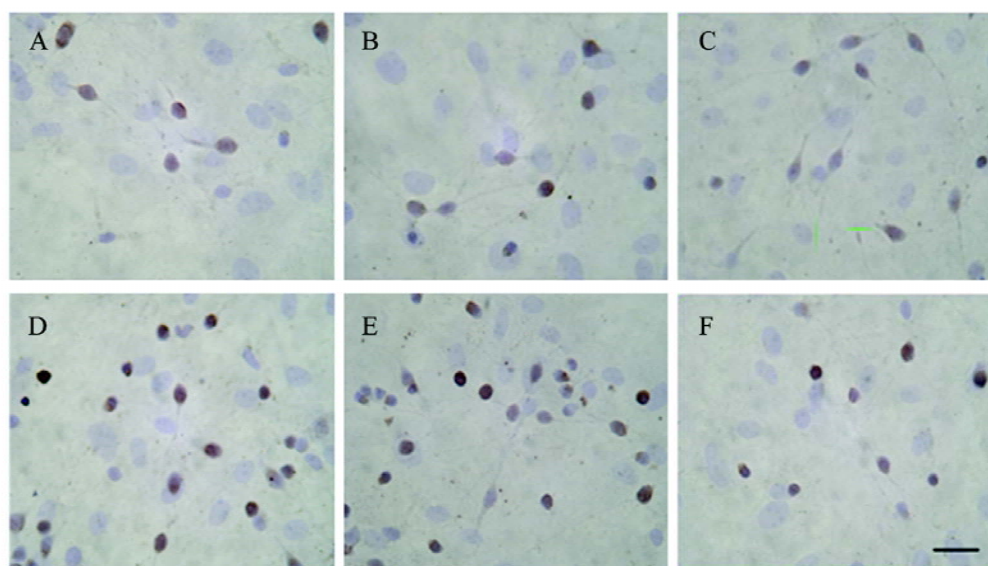


Fig. 1 Effects of $A\beta_{31-35}$ on caspase activities in cultured cortical neurons. $A\beta_{31-35}$ induced significant increases in the activities of caspase-3 and caspase-8. Neurons were treated with $A\beta_{31-35}$ (25 μmol/L) for 24 h, and caspase activities were measured using a spectrophotometer at 450 nm. *P < 0.05 *vs* the control.

increase was detected at 4 h, 8 h, 16 h and 24 h in $A\beta_{31-35}$ treatment, and the expression of p-c-Jun reached the peak at 8 h in $A\beta_{31-35}$ treatment. Phosphorylation of c-Jun was found to precede the onset of apoptosis, since characteristics of apoptosis were displayed at 24 h of $A\beta_{31-35}$ treatment (25 μmol/L) (data in publication).

To further confirm the involvement of JNK pathway in $A\beta_{31-35}$ neurotoxicity, the effect of SP600125, a specific inhibitor of JNK, on p-c-Jun expression in primary cultured cortical neurons was further investigated. As shown in Fig. 2, pretreatment with SP600125 (100 nmol/L) for 30 min could completely abolish $A\beta_{31-35}$ neurotoxicity. The increase in expression of p-c-Jun caused by $A\beta_{31-35}$ treatment for 8 h was significantly inhibited by pretreatment with SP600125 in cultured cortical neurons.



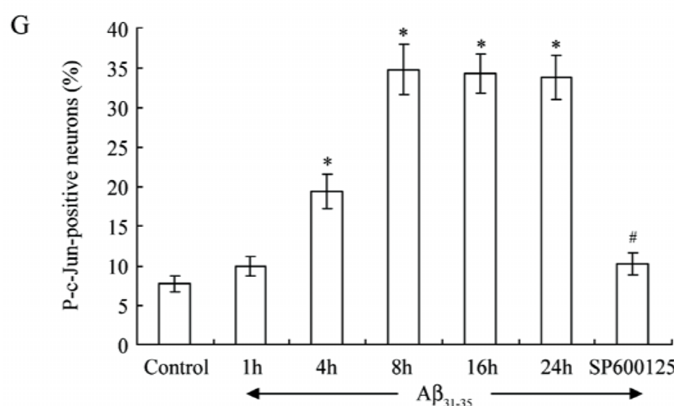


Fig. 2 Effects of $A\beta_{31-35}$ and SP600125 (100 nmol/L) on the expression of p-c-Jun. A-F were the representative photomicrographs in different groups. A: the control group; B: neurons treated with $A\beta_{31-35}$ (25 μmol/L) for 1 h; C: neurons treated with $A\beta_{31-35}$ (25 μmol/L) for 4 h; D: neurons treated with $A\beta_{31-35}$ (25 μmol/L) for 8 h; E: neurons treated with $A\beta_{31-35}$ (25 μmol/L) for 16 h; F: neurons were pretreated with SP600125 (100 nmol/L) and then treated with $A\beta_{31-35}$ (25 μmol/L) for 8 h; G: quantification of p-c-Jun-positive neurons. Comparison was conducted using one-way ANOVA. Values were from 5 separate experiments. *P < 0.05 *vs* the control, #P < 0.05 *vs* $A\beta_{31-35}$ treatment for 8 h. Scale bar, 100 μm.

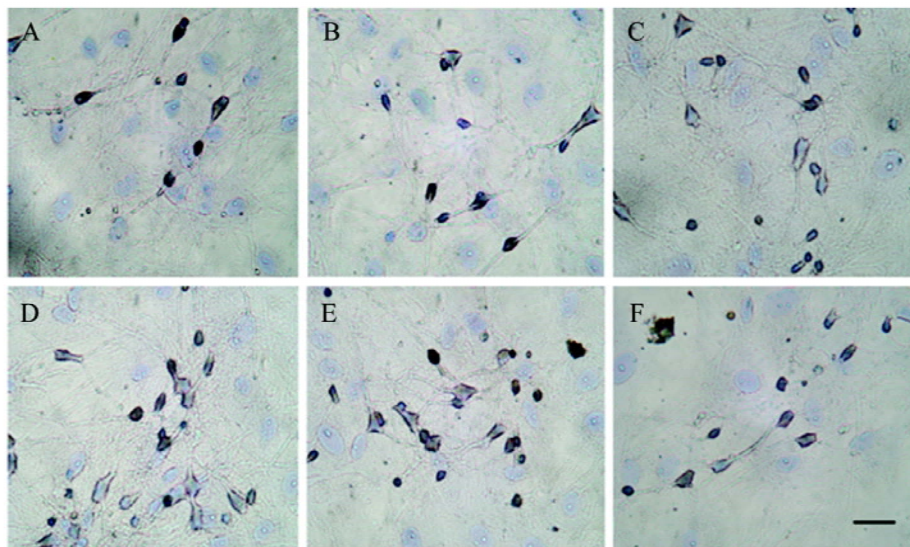


Fig. 3 **Effects of Aβ_{31-35} and SP600125 (100 nmol/L) on FasL expression, as determined by immunocytochemistry method using anti-FasL antibody. A-F were the representative photomicrographs in different groups. A: the control group; B: neurons treated with Aβ_{31-35} (25 μmol/L) for 1 h; C: neurons treated with Aβ_{31-35} (25 μmol/L) for 4 h; D: neurons treated with Aβ_{31-35} (25 μmol/L) for 8 h; E: neurons treated with Aβ_{31-35} (25 μmol/L) for 16 h; F: neurons were pretreated with SP600125 (100 nmol/L) and then treated with Aβ_{31-35} (25 μmol/L) for 8 h. G: quantification of FasL-positive neurons. Data were from 5 separate experiments, and analyzed using one-way ANOVA. *P < 0.05 *vs* the control, #P < 0.05 *vs* Aβ_{31-35} treatment for 8 h. Scale bar, 100 μm.**

3.3 Aβ_{31-35} induced a significant increase in the protein level of FasL The effect of Aβ_{31-35} on the expression of FasL in cortical neurons was also investigated. As shown in Fig. 3, treatment with Aβ_{31-35} led to a significant increase in the protein level of FasL, as assessed by immunocytochemistry method using anti-FasL antibody. The increase in FasL expression was observed at 8 h, 16 h and 24 h of Aβ_{31-35} treatment, indicating that it was a subsequent event to c-Jun activation. Moreover, the increase in expression of FasL caused by Aβ_{31-35} treatment for 8 h was significantly abolished by pretreatment with SP600125 (100 nmol/L) for 30 min in cultured cortical neurons.

4 Discussion

The accumulation of Aβ has been implicated as a cause of neuronal loss that occurs in AD brain. However, the underlying mechanisms are not well understood. The caspase family includes cysteine proteases and critical mediators of programmed cell death. Caspase-8 and caspase-9 are the initiators of the extrinsic pathway and the intrinsic pathway, respectively. Caspase-3 is one of the key executioners in the final phase of apoptosis, and is partially or completely responsible for the proteolytic cleavage of many proteins[4,15]. Caspase-associated apoptotic cell death has been reported in several neuronal cell types exposed to Aβ[13,16-19]. Here we demonstrate that Aβ_{31-35} induces a significant increase in the activities of both caspase-3 and caspase-8, accompanied with a significant increase in the percentage of apoptotic neurons in primary cultured cortical neurons with Aβ_{31-35} treatment (data not shown). These imply that Aβ_{31-35}-induced neuronal apoptosis is mediated by caspase-dependent extrinsic apoptotic pathway in primary cultured cortical neurons.

At present, the most widely studied extrinsic pathway involving caspase-3 is the JNK pathway. JNK has been shown to be activated by oxidative stress, NGF withdrawal, kainic acid, and Aβ[11-14]. To determine whether the JNK signaling pathway is involved in Aβ$_{31-35}$-induced apoptosis of cortical neurons, we asked whether Aβ$_{31-35}$ treatment would lead to the activation of the component of JNK pathway. The component c-Jun lies in the downstream of JNK pathway and has been shown to play a significant role in triggering neuronal apoptosis in a variety of cell types[11,12,20]. The effect of Aβ$_{31-35}$ on c-Jun expression was examined by immunocytochemistry method using anti-p-c-Jun antibody (at the site of Ser73), which could recognize the activated form of c-Jun. Results revealed that Aβ$_{31-35}$ treatment on cortical neurons led to the activation of c-Jun, accompanied with elevation of caspase-3 activity, implying that the activation of JNK pathway may be relevant to Aβ$_{31-35}$-induced cell apoptosis. This proposition is further supported by blocking JNK function. SP600125 (anthra[1,9-cd] pyrazol-6(2H)-one) is a recently developed specific inhibitor of JNK[21]. Hashimoto *et al.*[22] have reported that SP600125 can dose-dependently inhibit the anti-APP antibody 22C11-induced neuronal death in primary cultured neurons, at the concentrations between 10-100 nmol/L. Complete suppression could be achieved by 100 nmol/L SP600125 application. In the present study, the concentration of 100 nmol/L SP600125 was employed for the application of SP600125, and at this concentration, SP600125 could completely block the phosphorylation of c-Jun. These data indicate that JNK is specifically involved in Aβ$_{31-35}$ neurotoxicity.

Although a variety of JNK-c-Jun targets have been identified, several reports have suggested a significant role of FasL in neurodegenerative disease. In an animal model of focal cerebral ischemia, expressions of FasL and Fas are induced in neurons undergoing apoptosis[20,23]. In several neurodegenerative diseases such as Parkinson's disease (PD) and Down syndrome, the up-regulation of Fas expression was detected in dying neurons[24,25]. Here FasL expression could be induced by Aβ$_{31-35}$ treatment, and the disruption of JNK function led to a marked decrease in FasL expression.

In summary, the present study suggests that Aβ$_{31-35}$ may activate JNK, which in turn activates the downstream c-Jun, FasL and caspase-dependent extrinsic apoptotic pathways, ultimately leading to neuronal apoptosis. Moreover, the question of how extracellular Aβ$_{31-35}$ induces the intracellular JNK activation may also be of interest for researchers to fully understand the mechanism.

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JNK 激活的外源性凋亡途径介导 $A\beta_{31-35}$ 诱导的神经元凋亡

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摘要：目的 探讨JNK信号通路在 $A\beta_{31-35}$ 诱导的神经元凋亡过程中的作用。**方法** 经老化处理的 $A\beta_{31-35}$ (终浓度为25 μmol/L)制备AD细胞模型，采用生物比色法检测caspase-3和caspase-8的活性。采用免疫细胞化学技术观察不同时间点磷酸化c- Jun (p-c-Jun) 及Fas ligand (FasL) 蛋白的表达情况，并用IPP11.0图像分析软件进行定量分析。**结果** $A\beta_{31-35}$ 孵育24 h能显著提高神经元内caspase-3和caspase-8的活性。$A\beta_{31-35}$ 孵育4 h时p-c-Jun蛋白表达水平开始升高，在8 h升高最显著，呈现一定的时间依赖性；JNK特异性抑制剂SP600125能抑制 $A\beta_{31-35}$ 对p-c-Jun蛋白表达的诱导作用。$A\beta_{31-35}$ 孵育8 h时出现FasL蛋白表达的升高，而SP600125则能抑制这一作用。**结论** JNK激活的外源性凋亡途径在 $A\beta_{31-35}$ 诱导的神经元凋亡过程中发挥一定的作用。
关键词：$A\beta_{31-35}$；神经毒性；caspase；JNK 通路